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Multiple factors conferring high radioresistance in insect Sf9 cells

I-Cheng Cheng[1,2], How-Jing Lee[1] and T. C. Wang[2,*]

[1]Department of Entomology, National Taiwan University, Taipei, Taiwan and [2]Institute of Cellular and Organismic Biology, Academia Sinica in Taiwan, Taipei, Taiwan

Sf9, a lepidopteran cell line isolated from the fall armyworm, *Spodoptera frugiperda*, was shown to be significantly more resistant to growth inhibition and apoptosis induction effects of x-ray irradiation than several human cell lines of different origins. The single-cell electrophoresis technique revealed that Sf9 cells showed lower x-ray irradiation-induced DNA damage as well as better efficiency at repairing these damages. In addition, Sf9 cells were lower in both background and x-ray irradiation-induced intracellular oxidative stress, in which the higher intracellular level of reduced glutathione seemed to play a major role. The significance of oxidative stress in determining the radioresistance of Sf9 cells was confirmed by their being more resistant to hydrogen peroxide while equally susceptible to other non-reactive oxygen species of *N*-nitroso alkylating agents when compared with a human cell line. Although the Sf9 and human cell lines were equally susceptible to the lethal effects of *N*-nitroso alkylating agents, the components of DNA damage-induced and the repair enzymes involved significantly differ. This phenomenon is also discussed in this report.

Introduction

Ionizing radiation is generally considered to be a harmful agent to organisms. Individual mammals exposed to high doses of ionizing radiation are known to be at risk of developing cancer (1). According to recent reviews, ionizing radiation at low doses can also cause cancer due to its late effect (2–4). Exposure to ionizing radiation has been demonstrated to result in a self-sustaining and long-lasting process of clastogenesis in the human body, which may exceed the ability of the DNA repair system to deal with it, eventually leading to cancer (5,6). Minimizing human exposure and increasing resistance to ionizing radiation are important issues for human health. Ionizing radiation, on the other hand, serves as a powerful and prevalent clinical tool in cancer therapy. Development of cellular resistance to ionizing radiation, however, is a significant component of radiotherapy failure (7). Therefore, elucidating resistance mechanisms to ionizing radiation is not only essential for protecting humans from ionizing radiation but also significant in efforts to overcome resistance problems encountered clinically.

The responses of an organism to the lethal effects of ionizing radiation considerably vary among different species. Compared to humans, insects are generally known to be more resistant to ionizing radiation (8,9). Various mechanisms have been proposed for the higher radioresistance of insects. However, it is difficult to compare individual humans and insects due to their enormous differences. Investigations using cellular models therefore provide alternative approaches to scrutinize this issue. Using cell cultures, the relative radioresistance levels of cells isolated from different insect orders were found to correspond with those of whole insects (10–12). It seems that the high radioresistance in insect cells is relevant to the true character of insect individuals. Confirmation of an organism's resistance to irradiation at the cellular level initiated a series of investigations into the mechanisms in cultured insect cells. The intrinsic nature of radioresistance in insect cells, especially in those of lepidopteran insects, provides a useful working model to understand how organisms like insects can tolerate such high dosages of irradiation. Previous studies on a cell line isolated from a lepidopteran, *Trichoplusia ni*, TN368, concluded from the results of unscheduled DNA synthesis experiments that the higher DNA repair efficiency might account for its superior radioresistance over mammalian cells (9,13,14). Analyses on the dose–response curves using the same insect cell line reached a similar conclusion that the repair process might be induced or activated after irradiation treatment (15). The lower DNA damage, which is attributable to the ability to carry out DNA repair more efficiently, appeared to be a key factor in the higher survival of irradiated insect cells. This phenomenon which was mostly observed in experiments with TN368 cells was recently confirmed in a study using another lepidopteran cell line, Sf9, which was isolated from the fall armyworm, *Spodoptera frugiperda* (16). In previous documents, the better efficiency of DNA repair was recognized as the predominant factor accounting for the higher radioresistance in insects. Discussion of the involvement of other mechanisms is rare. In fact, however, in addition to DNA damage, ionizing radiation also generates intracellular reactive oxygen species (ROS) (17–21), including superoxide anions, hydrogen peroxide and hydroxyl radicals, which are deleterious to various cellular components such as DNA, mitochondria, proteins and cell membranes (22,23). The mechanisms capable of quenching intracellular ROS might also play significant roles in determining the higher radioresistance in insects and/or insect cells. In order to elucidate the radioresistance mechanism in greater detail, we investigated the x-ray-induced cellular stress, including intracellular ROS and DNA strand breaks, and their repair in insect Sf9 cells. Several cell lines of human origin were included in order to make comparative studies. Factors and mechanisms of the radioresistance of insect cells were analysed and are discussed in this communication.

Materials and methods

Cell cultures

The insect cell line, Sf9, originally established from the fall armyworm (*S. frugiperda*) of the Lepidoptera, was a kind gift from Prof. C. H. Wang at

*To whom correspondence should be addressed. Tel: + 886 2 27899540; Fax: +886 2 27858059; Email: tcwang@sinica.edu.tw

National Taiwan University, Taipei, Taiwan. Sf9 cells were maintained in Sf-900 II serum-free medium (GIBCO BRL, Carlsbad, CA, USA) at 28°C in a humidified incubator. Four human leukaemia cell lines (promyelotic HL-60 and NB4 cells and monocytic THP-1 and U937 cells), a human gastric carcinoma cell line (SC-M1) and a bladder carcinoma cell line (NTUB1) were cultured in RPMI 1640 (GIBCO BRL) containing 10% foetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mM of L-glutamine and 2 mg/ml of sodium bicarbonate and kept at 37°C in a humidified incubator with 5% CO_2 in the dark. Cells for each experiment were limited to those which had been thawed for <1 month.

Irradiation

Cells were seeded in six-well dishes, with each dish containing 1×10^5 cells, and incubated overnight before x-ray irradiation. Different single log doses were given as 5, 10 and 20 Gy. The irradiation was carried out at room temperature using a Torrex 150D x-ray machine (EG&G; Astrophysics Research, Long Beach, CA, USA) operated at 134 kV and 4 mA with a 1.2-mm beryllium filter at a dose rate of 6 Gy/min.

Growth assay

The growth condition of cells was measured by estimating the cell numbers using UV absorption at 260 nm as DNA contents as previously proposed by Chang (24). In brief, cells in each treatment were harvested in a 15-ml tube. The media were removed by centrifugation, and then cells were washed twice in phosphate-buffered saline (PBS). The DNA of cell pellets was fixed in icy methanol at 4°C for 20 min. Cells were air-dried by inverting an open tube. DNA was re-suspended in 1 ml of 0.2 N sodium hydroxide for 2 h. The absorption at 260 nm for each cell solution was measured with a U2000 spectrophotometer (Hitachi, Tokyo, Japan). The percentage of growth fraction in each sample was calculated by dividing the absorbance value of treated cells by that of the control group. The growth assay of x-ray cytotoxicity was carried out after irradiation exposure for 3 days, with other agents being administered for 24 h for the subsequent growth assay.

Apoptosis assay

In the DNA ladder assay, apoptotic DNA from insect and human cells treated with x-ray irradiation for 3 days was selectively extracted using a DNA ladder assay kit (Suicide Track; CALBIOCHEM, Darmstadt, Germany). DNA was separated on a 2% agarose gel in TBE buffer [89 mM Tris–borate and 2 mM ethylenediaminetetraacetic acid (EDTA)] at 100 V. Ladders of DNA were visualized by staining with 10 μg/ml ethidium bromide.

In the annexin V assay, cells were double stained with annexin V–fluorescein isothiocyanate and propidium iodide provided by apoptosis detection kit (CALBIOCHEM). With filtering through a 40-μm nylon mesh, annexin V positivity was determined by Epics XL-MCL flow cytometry with EXPO ADC XL4 Color software, and data were analysed using EXPO32 ADC Analysis (Beckman Coulter, Fullerton, FL, USA).

DNA damage assay

Single-cell electrophoresis, also known as the alkaline comet assay, was used to measure DNA strand breaks. In brief, fully frosted slides were warmed on a 65°C hot plate. Eighty microlitres of 1% normal melting gel (GIBCO BRL) in PBS were dropped onto the slides and immediately covered with a 24×40-mm cover glass. Slides were transferred to an ice-cold 4°C plate to allow the gel to solidify. Cover glasses were carefully removed in chilled water to allow the undercoated gel to set. Treated cells were washed twice in PBS. Fifty microlitres of the cell suspension (containing 5×10^5 cells/ml) plus 250 μl 1.2% low melting agarose (GIBCO BRL) in PBS were equally mixed by pipetting. Eighty microlitres of the cell–gel mixtures were also dropped onto slides which had been pre-warmed over a 37°C hot plate. The second layers were set in the same manner as the first ones. At this point, procedures were carried out in dim light to prevent non-irradiation DNA damage. Slides were carefully immersed in chilled comet lysis buffer (2.5 M sodium chloride, 100 mM sodium EDTA and 10 mM Tris base, with the pH adjusted to 10 with solid sodium hydroxide; 10% dimethyl sulphoxide and 1% Triton X-100 were added just before use) at 4°C overnight. The next day, slides were denatured in electrophoresis (denatured) buffer containing 0.3 M sodium chloride (pH 13.4) and 1 mM sodium EDTA for 20 min. Electrophoresis was run at 25 V and 300 mA (the voltage was fixed and current was adjusted based on the buffer volume) for 20 min. Slides were briefly rinsed in double-distilled water and then transferred to 0.4 M Tris–HCl (pH 7.5) for re-naturing. Samples were kept in buffer and analysed within 48 h.

To observe the comets, slides were stained with 10 μl of 20 μM YOYO-1 [Molecular Probes, Eugene, OR, USA; in glycerol/0.4 M sodium EDTA, pH 7.4 (50/50), and 0.1% 8-hydroxyquinoline], covered with a cover glass and then immersion oil (Immersol 518F; Zeiss, Oberkochen, Germany) was applied. Comets were observed under a fluorescence microscope (Zeiss, Oberkochen, Germany; with a shortwave pass filter of 450–490 nm, a chromatic beam splitter of 510 nm and a long-pass filter of 520 nm) with ×250 amplification. Images of 100 individual cells per treatment randomly selected from three slides were recorded with a digital camera (DCS-420; Kodak, Rochester, NY, USA). Migration of DNA from the nucleus in each cell was expressed by the parameter of the tail moment (a product of the tail length and intensity) (25) and measured with the COMET Assay III program (Perspective Instruments, Suffolk, UK). The handling protocols mainly followed those described by Liu and Jan (26).

Analysis of intracellular oxidative stress

The intracellular oxidative stress assay took advantage of the characteristic of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes) to highly penetrate cells. DCFH-DA is rapidly taken up by cells, and the diacetate (DA) part is removed, leaving the surplus dichlorodihydrofluorescein (DCFH) as a fluorescent reporter. DCFH is then oxidized by intracellular peroxides to dichlorofluorescein (DCF), which fluoresces when excited by a 488-nm wavelength. For the assay, cells were incubated in medium containing 10 μM DCFH-DA for 30 min after 20 Gy of x-ray irradiation or 0.8 mM hydrogen peroxide treatment. The DCF fluorescence of each cell population was obtained by flow cytometry. The DCF intensity induced by either x-ray irradiation or hydrogen peroxide was normalized to the untreated control in each cell type.

Measurement and depletion of glutathione

Intracellular reduced glutathione (GSH) levels were measured using a commercially available glutathione assay kit (CALBIOCHEM). In brief, cells in each treatment were collected and homogenized in 400 μl 5% (w/v) metaphosphoric acid (Merck, Darmstadt, Germany) before two rounds of washing in PBS. The supernatant was obtained by centrifugation at 15 000 × *g* at 4°C for 10 min. Two hundred microlitres of supernatant were added to the working solution offered with the vortexing kit. The reaction was incubated in the dark at room temperature for 10 min before measuring the absorbance at 400 nm with a U2000 spectrometer (Hitachi). The GSH level in each treatment, expressed as nanomoles per milligram of protein, was obtained by comparison to a standard curve of known GSH concentrations that ranged 20–100 μM and was finally normalized to the protein concentrations in the cell extracts determined according to the method derived from Bradford (27) using BSA ranging 1–8 μg/ml as the standards.

Buthionine sulfoximine (BSO), an agent which blocks GSH synthesis by inactivating γ-glutamylcysteine synthetase, was employed. Insect and human cells were cultured in medium containing BSO for 24 h to deplete GSH. To treat x-ray-irradiated insect cells with GSH depletion, BSO was replaced by fresh Sf-900 II serum-free medium.

Preparation of cellular extracts

Cells were harvested and washed twice in PBS and washed another time in Hank's buffered salt solution (HBSS) and then were lysed in 150 μl lysis buffer [containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.4), 0.5% (v/v) Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml benzamidine; 10 μg/ml each of leupeptin, aprotinin and pepstatin A was added just before use] for 2 h at 4°C. After centrifugation (16 000 × *g* for 5 min), the supernatants were collected. Protein concentrations in the cell extracts were determined based on the procedure of Bradford (27).

Immunoassay

Protein extracts (50 μg) mixed with 2× protein sample buffer [0.125 M Tris (pH 6.8), 4% sodium dodecylsulfate (SDS), 20% glycerol, 0.02% bromophenol blue and 0.5% 2-mercaptoethanol] were heated at 95°C for 5 min. Afterward, they were separated on 10% SDS–polyacrylamide gel (PAGEs) at 100 V and transferred to polyvinylidene fluoride (PVDF) membranes at 20 V for 15 min. After blocking with 5% milk buffer for 1 h, the blots were incubated with a monoclonal antibody at 4°C overnight. After three washes in tris-buffered saline Twen-20 (TBST) [125 mM NaCl, 25 mM Tris (pH 8.0) and 0.1% Tween 20], blots were probed with a horseradish peroxidase-conjugated secondary antibody for 1 h and then washed in TBST three times. The protein signal of interest was visualized by chemiluminescence (Pierce, Rockford, IL, USA). Besides antiserums against actin (Chemicon, Temecula, CA, USA) and O6-methylguanine DNA methyltransferase (MGMT) (Lab Vision, Suffolk, UK), other commercial antibodies were purchased from BD Biosciences (San Diego, CA, USA).

Results

Unbiased estimation of cell numbers using UV absorbance at 260 nm

Estimation of cell numbers through an approximation of the DNA amount was previously proposed by Chang (24). The

total DNA amount of cells contained in each sample was determined by the absorbance at 260 nm. By dividing the absorbance value of the treatment group by that of the control group, the relative frequency of cell growth in each treatment was estimated. An assay on growth inhibition using this protocol was established for mammalian cell cultures (28,29). In order to verify if this protocol would also be appropriate for insect Sf9 cells in culture, a series of control treatments with cell numbers varied from 1- to 10 × 10^5 were sampled, and the absorbance at 260 nm was determined using a Hitachi 2000 spectrophotometer (Figure 1). A significant linear correlation between the cell number and absorbance at 260 nm was found with an r^2-value of 0.999. This indicates that the protocol proposed by Chang (24) provides an unbiased estimate of the number of Sf9 cells. Application of this protocol is especially expedient for the growth inhibition assay in Sf9 cells since Sf9 cells grow in culture by loosely attaching to the bottom of the culture vessel and are thereby unable to form firm colonies.

Insect Sf9 cells are more resistant to x-ray irradiation than human cells

Sf9 and six human cell lines were irradiated with 0, 5, 10 and 20 Gy x-ray irradiation. After cessation of x-ray irradiation treatment, cells were incubated for 72 h before being assayed for growth inhibition using the protocol described in the previous section. The dose that induced 50% cell growth inhibition (LD_{50}) was estimated using the CompuSyn quantitation program (ComboSyn, Paramus, NJ, USA). Human promyelocytic NB4 cells were the most sensitive cell line to x-ray irradiation among the cell lines investigated in this study, with an LD_{50} value of 3.7 Gy. Therefore, the comparative resistance ratio of each cell line to x-ray irradiation was calculated by dividing the LD_{50} of each cell line by that of NB4. The resistance ratio among cell lines showed a descending order as follows: Sf9 > SC-M1 > THP-1 > NTUB1 > HL-60 > U937 > NB4 (Figure 2). Insect Sf9 cells were much more resistant to x-ray irradiation than any of the remaining cell lines of human origin. The growth inhibition induced by x-ray irradiation in Sf9 cells was almost non-detectable up to doses as high as 20 Gy. In those cells of human origin, on the other hand, cell growth was inhibited by x-ray irradiation in a dose-dependent manner at dose levels of <20 Gy. Among human cell lines, gastric SC-M1 cells were the most and NB4 cells were the least resistant to x-ray irradiation. These two human cell lines were picked as references for further investigations to help elucidate the x-ray irradiation resistance mechanism in Sf9 cells. Sf9 cells, with an LD_{50} of x-ray irradiation of 49.7 Gy, were 13.4 and 3 times as resistant as NB4 and gastric SC-M1 cells, respectively. The differential growth inhibitory effect of x-ray irradiation among Sf9, SC-M1 and NB4 cells was confirmed through microscopic observations (data not shown). There was no noticeable change in cell numbers of Sf9 cells after x-ray irradiation of up to 20 Gy. Densities of SC-M1 and NB4 cells, in contrast, distinctly decreased as the dose of x-ray irradiation increased.

X-ray irradiation induces apoptosis in human but not insect Sf9 cells

Induction of apoptosis has been demonstrated to be associated with ionization-induced cell death (30–37). Two approaches which have been widely used to assess the induction of apoptosis, including the DNA ladder assay and annexin V staining technique, were used to investigate the mechanism of cell death in Sf9, SC-M1 and NB4 cells. Formation of fragmented DNA, one of the typical apoptotic characters detected by the DNA ladder assay, was observed in both SC-M1 and NB4 cells after x-ray irradiation (Figure 3). Sf9 cells, on the other hand, did not show induction of DNA fragmentation. These results were further confirmed by annexin V staining, a protocol that detects the induction of apoptosis at an early stage (38,39). A dose-dependent increase in the frequency of annexin V-stained cells was observed in both SC-M1 and NB4 cells after x-ray irradiation (Figure 4). In these two human cell lines, treatment with x-ray irradiation at a dose of as low as 5 Gy was sufficient to significantly induce apoptosis. X-ray-irradiated Sf9 cells, on the contrary, did not show significant apoptosis induction, even at as high a dose as 20 Gy.

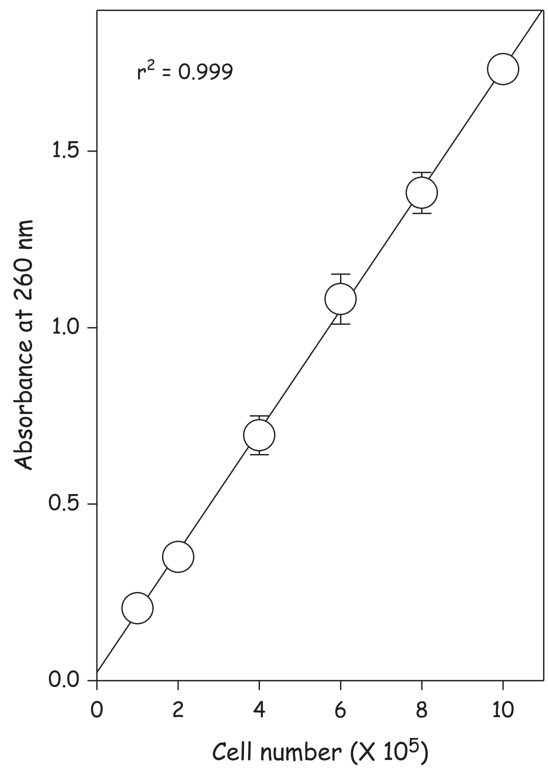
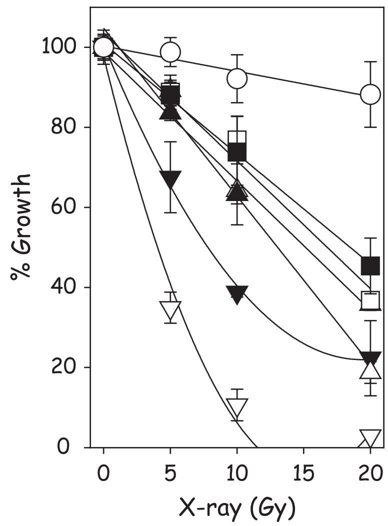


Fig. 1. Linear correlation between cell numbers and the absorbance at 260 nm in insect Sf9 cells.

Insect Sf9 cells are more resistant to x-ray irradiation-induced DNA damage

DNA damage induced by x-ray irradiation was investigated using the single-cell gel electrophoresis technique, which is also known as the comet assay. The induction of DNA strand breaks was indicated by the level of the tail moment measured from each assayed nucleus. Examination immediate after exposure to x-ray irradiation found that a significant increase in the tail moment was found in NB4 cells, indicating a substantial induction of DNA damage (Figure 5, lower panel). The induction of DNA damage, as shown by the median of the tail moment in the comet assay, increased 50-, 70- and 80-fold as much as in the control treatment in NB4 cells when exposed to 5, 10 and 20 Gy x-ray irradiation, respectively. In Sf9 cells, the induction of DNA damage was much lower than in NB4 cells, respectively, showing only



Cell lines	LD50 (Gy)	Resistance level
○ Sf9	49.7	13.4
■ SC-M1	18.2	4.9
□ THP-1	16.3	4.4
▲ NTUB1	13.9	3.8
△ HL-60	11.7	3.2
▼ U937	8.0	2.2
▽ NB4	3.7	1.0

Fig. 2. Cytotoxicity of x-ray irradiation to insect cells and seven human cell lines. Left panel: dose-responsive curves for each cell line; right panel: estimation of the radioresistant ratio in each cell line to NB4 cells based on the 50% growth inhibition dosage (LD_{50}) values.

3-, 5- and 15-fold increases in the median of the tail moment for the same treatment series. DNA damages induced in SC-M1 cells were in between those two cells, which is relevant to its intermediate cytotoxicity response shown earlier in this report. Differences in x-ray-induced DNA damage among the three cell lines were confirmed by the morphology of damaged nuclei (Figure 5, upper panel). Most of the damaged nuclei induced by x-ray irradiation in NB4 cells showed a morphology of a longer tail and a smaller head than those in Sf9 and SC-M1 cells, indicating a greater loss of broken DNA strands migrating to the tail. Among insect and human cell lines, levels of DNA damage were positively correlated with that of growth inhibition (r^2 = 0.95, Figure 6, left panel) and apoptosis induction (r^2 = 0.87, Figure 6, right panel). This possibly suggests that x-ray irradiation-induced DNA damage was strongly associated with cell death. In SC-M1 and Sf9 cells, induction of DNA damage by exposure to 10 Gy x-ray irradiation did not significantly differ, as indicated by the close median values of the tail moment, at 25.8 versus 21.2 and 24.7 versus 29.6, respectively, in the two independent experiments shown in Figures 5 and 7. Previous results shown in Figures 2–4, however, revealed that treatment with 10 Gy of x-ray irradiation induced much greater growth inhibition and apoptosis induction in SC-M1 than in Sf9 cells. The inconsistency between DNA damage and growth inhibition might have been due to a differential efficiency in repairing damaged DNA.

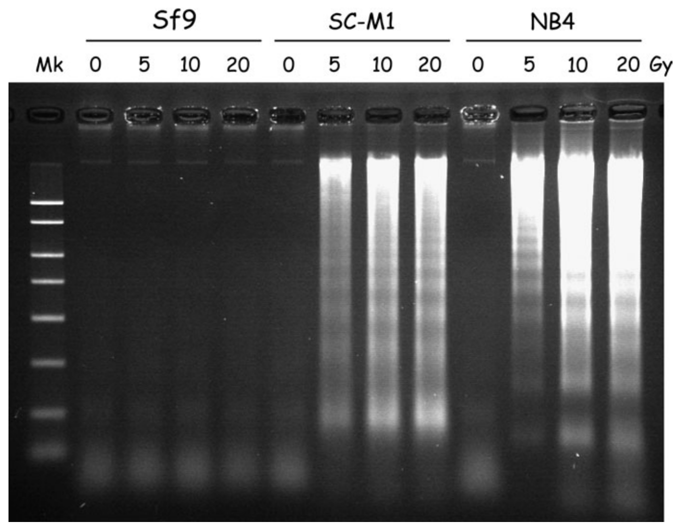


Fig. 3. DNA ladder assay showing x-ray irradiation-induced DNA fragmentation in the insect Sf9, human gastric SC-M1 and human promyelocytic NB4 cell lines.

Insect Sf9 cells are also more efficient in repairing DNA damage than human cells

In order to investigate the DNA repair efficiency of SC-M1 and Sf9 cells, the tail moment was measured in cells harvested at the end of treatment (0 h) and 2 and 24 h after treatment cessation (Figure 7). DNA damages in both Sf9 and SC-M1 cells were significantly reduced at 2 h after x-ray irradiation had been stopped (Figure 7, left panel). However, the DNA repair seemed to be more efficient in Sf9 cells. The median tail moment in Sf9 cells decreased from 24.7 to 5.6, while that in SC-M1 cells decreased from 29.6 to 10.3. In addition, induction of the tail moment in SC-M1 cells seemed to be divided into two distinct groups of low and high levels. The group of those with a high tail moment consisted of ~7% of the total SC-M1 cell population, showing that tail moment measurements ranged 51–99, while those of the remaining 93% were <45. This indicates that the response of SC-M1 cells to x-ray irradiation is heterogeneous. When the post-irradiation period was extended more to 24 h, the frequency of nuclei with a high tail moment, measuring between 82 and 163, increased to ~28%. Based on the observation that the frequency of cells with a high tail moment was close to the frequency of apoptotic cells induced in the late stage, we suggest that those cells with a high tail moment were likely to undergo apoptosis thereafter. SC-M1 cells with a low tail moment were capable of repairing the DNA damage and, consequently, showed that their nuclear morphology was as intact as those in the control group. The co-existence of nuclei with high and low tail moments is demonstrated in the right panel of Figure 7. The heterogeneous response to x-ray irradiation found in SC-M1 cells was not observed in Sf9 cells. Sf9 cells maintained low DNA damage

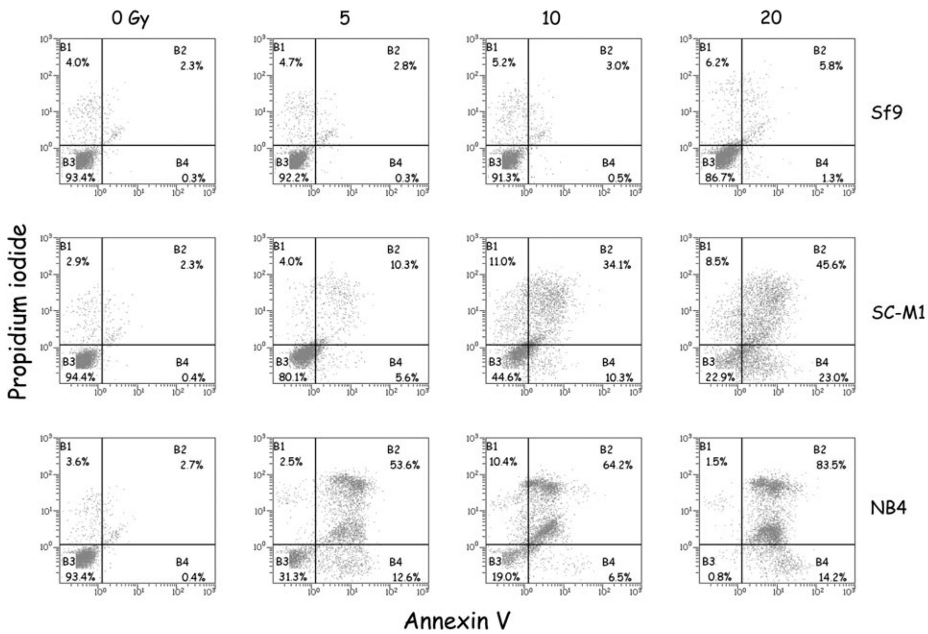


Fig. 4. Flow cytometric assay of x-ray irradiation-induced apoptosis using the annexin V–fluorescein isothiocyanate staining technique in the insect Sf9, human gastric SC-M1 and human promyelocytic NB4 cell lines.

induction despite the increase in the post-irradiation incubation period. This implies that in insect cells, damaged DNA is repaired more efficiently than in human cells. This result also provides an explanation for the conflicting phenomena that x-ray irradiation induced DNA damage at a similar level in insect Sf9 and human SC-M1 cells, while it was more toxic to human SC-M1 cells.

Insect Sf9 cells had lower background and induced oxidative stresses

In addition to inducing DNA strand breaks, x-ray irradiation is also known to increase intracellular ROS (17–19). We therefore attempted to elucidate if the intracellular ROS level, either background or induced, played a role in determining the differential resistance to x-ray irradiation between insect and human cells. The background level and alterations in intracellular ROS levels after x-ray irradiation were estimated by measuring the relative DCF intensity with a Coulter Epics XL-MCL flow cytometer. NB4 cells had the highest background ROS level among these three cell lines, with a relative DCF intensity 1.7 and 4.7 times as high as in SC-M1 and Sf9 cells, respectively (Figure 8). The ROS level in SC-M1 was 2.7 times as high as that in Sf9 cells. The background intracellular ROS level in insect Sf9 cells was much lower than those in human cells. The ROS level induced by x-ray irradiation in human cells was also higher than that in insect cells (Figure 9, left panel). The increase in DCF intensity with 20 Gy of x-ray irradiation in NB4 and SC-M1 cells was each ~2.4-fold; while in Sf9 cells, it was only 1.7-fold. Similar results were obtained in the experiment which directly applied H_2O_2 (Figure 9, right panel). Treatment with 0.8 mM H_2O_2 increased the DCF intensity by ~1.7-fold in Sf9 cells, which was similar to the level induced by x-ray irradiation. The increase in the DCF intensity by the same treatment was 42.4-fold in NB4 cells and 5.8-fold in SC-M1 cells. The increase in the ROS level in SC-M1 cells was intermediate between those of NB4 and Sf9 cells, which is relevant to the pattern of growth inhibition induced by H_2O_2 (Figure 10). With treatment with 0.4 mM H_2O_2, growth of >70% of NB4 cells was inhibited, while that was seen in only 25% of Sf9 cells. The same treatment induced 35% growth inhibition in SC-M1 cells, which was intermediate between NB4 and Sf9 cells. As the concentration of H_2O_2 in the treatments approached 1.6 mM, inhibition of cell growth in both SC-M1 and NB4 cells increased to 85–90%, which still allowed 60% Sf9 cells to continue to grow. Therefore, Sf9 cells not only had a lower background but also lower x-ray irradiation- and H_2O_2-induced ROS levels compared to human cell lines. These results to some extent account for the higher resistance level of Sf9 cells to x-ray irradiation and H_2O_2.

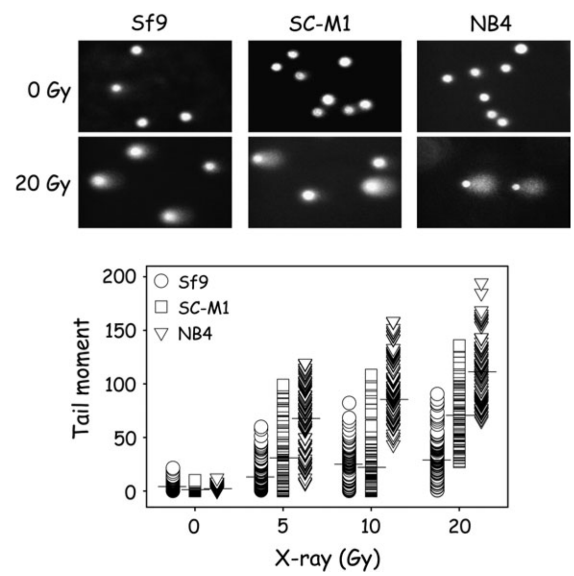


Fig. 5. Comet assay of x-ray irradiation-induced DNA damage in the insect Sf9, human gastric SC-M1 and human promyelocytic NB4 cell lines. Upper panel: photographic observations (×250); lower panel: relative tail moment induced in nuclei of treated cells.

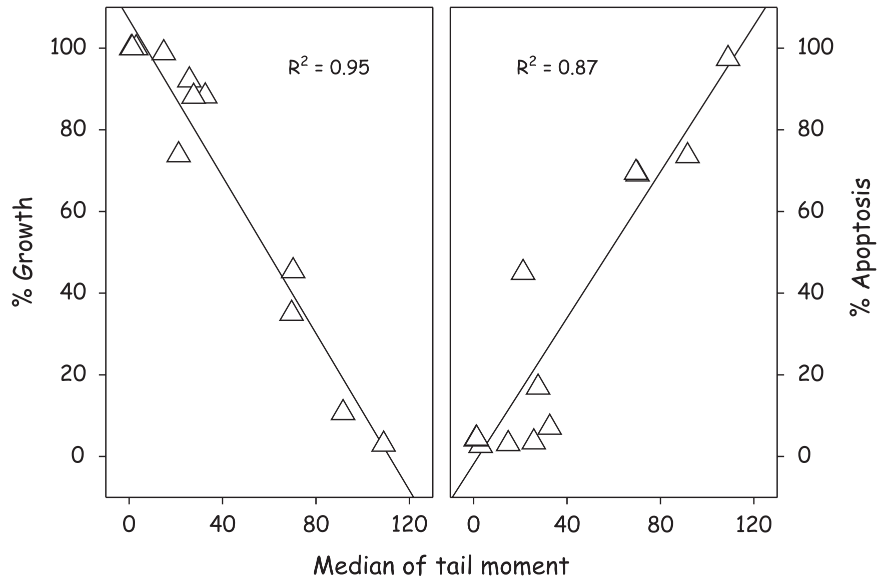


Fig. 6. Correlations of the induced tail moment with growth inhibition (left panel) and with apoptosis induction (right panel).

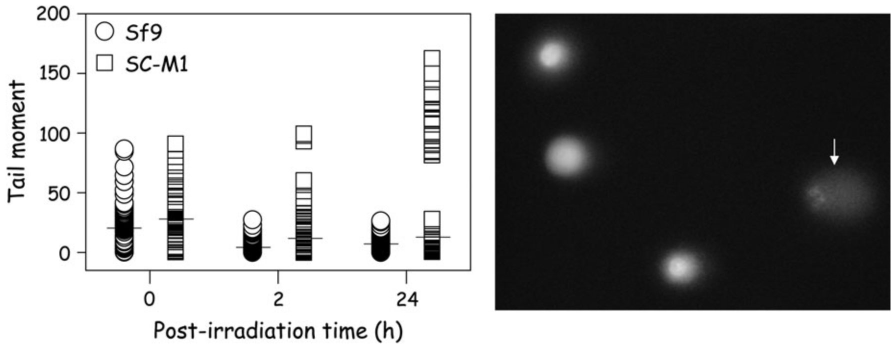


Fig. 7. Comparisons of the DNA damage repair ability between insect Sf9 and human SC-M1 cells. Left panel: cells treated with 10 Gy of x-ray irradiation were harvested for the comet assay at the end of treatment and 2 and 24 h after treatment cessation. Right panel: photograph (×250) showing the inferior DNA repair ability of human SC-M1 cells after 24 h of recovery following cessation of x-ray irradiation. The long nuclear tail remaining 24 h after treatment cessation, as indicated by the arrow, implies a failure by SC-M1 cells to repair x-ray irradiation-induced DNA damage.

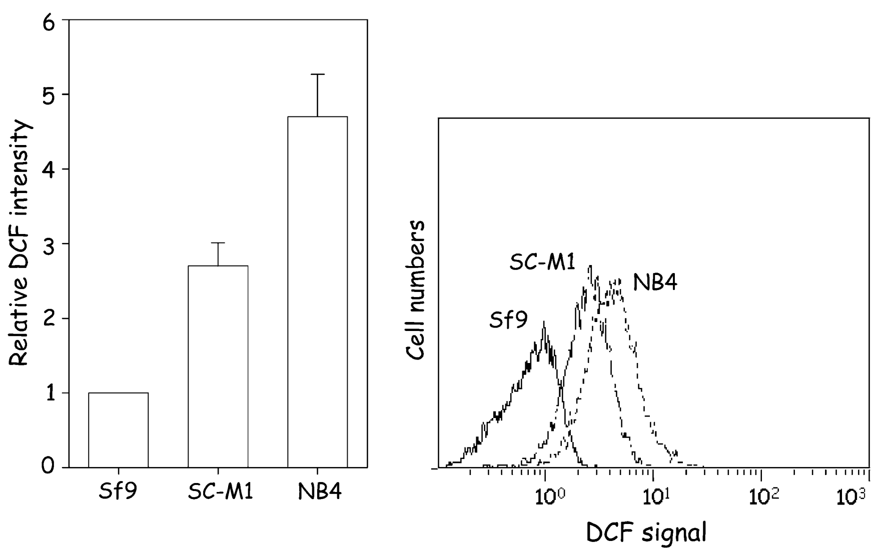


Fig. 8. Comparisons of basal intracellular oxidative stress levels expressed by the relative DCF intensity among the insect Sf9, human gastric SC-M1 and human promyelocytic NB4 cell lines. Left panel: histogram summary; right panel: flow cytometric illustration.

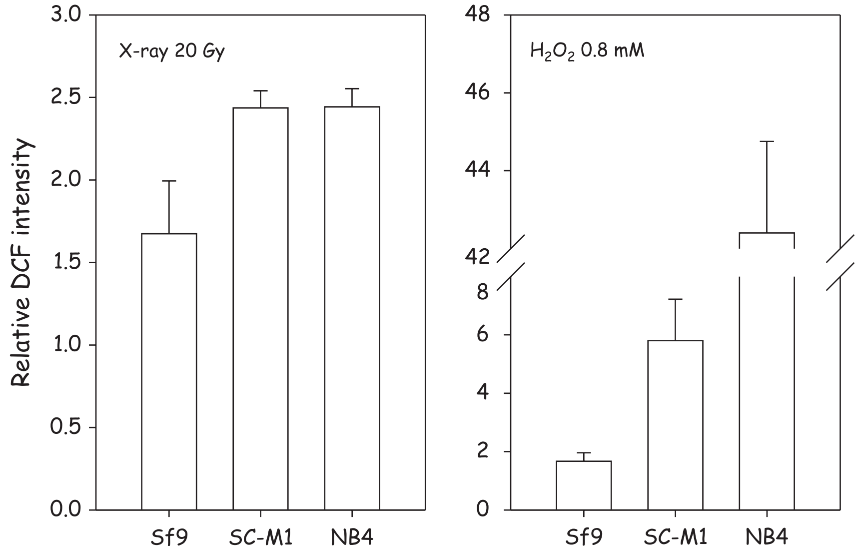


Fig. 9. Comparisons of x-ray irradiation (left panel)- and hydrogen peroxide (right panel)-induced intracellular oxidative stress levels expressed by the relative DCF intensity among the insect Sf9, human gastric SC-M1 and human promyelocytic NB4 cell lines.

Depletion of GSH enhanced growth inhibition of irradiated insect Sf9 cells

Reduction of GSH is one of the most important mechanisms in the protection of cellular macromolecules from being damaged by intracellular ROS (40). The higher background GSH levels of insect cells have been attributed to their resistance to mutagenic insults (41). Our investigation of Sf9 cells also obtained similar results. The background intracellular GSH level in Sf9 cells was found to be ~82.4 nmol/mg protein, which was twice as high as the 36.4 nmol/mg protein in SC-M1 cells and five times as high as the 17.2 nmol/mg protein in NB4 cells (Figure 11). Depletion of GSH synthesis by BSO revealed that treatment with 10 μM BSO efficiently inhibited GSH synthesis in SC-M1 and NB4 cells. While in Sf9 cells, >70% of GSH synthesis was resistant to BSO treatment at this dose level. When the BSO concentration was increased to 50 μM, the GSH level decreased from 71 to 26% in Sf9 cells. Further increasing the BSO dosage up to 400 μM did not completely remove intracellular GSH either. In the treatment with such an extremely high BSO dosage, 20% of GSH activity was still detected in Sf9 cells. Treatments with 10 and 50 μM BSO in Sf9 cells, although, respectively, inhibiting 29 and 74% of the GSH activity, were not cytotoxic to this cell line (Figure 12). Therefore, BSO at those dosages were chosen to inhibit GSH synthesis during the experiment with Sf9 cells. BSO treatment did enhance the cell growth inhibition induced by x-ray irradiation in Sf9 cells (Figure 12). Treatment with 10 μM BSO significantly enhanced the cell growth inhibition of Sf9 cells induced by 10 and 20 Gy x-ray irradiation ($P < 0.05$, *t*-test). In treatment with 50 μM BSO, Sf9 cells became more vulnerable to x-ray irradiation. The effective dose of x-ray irradiation that significantly inhibited cell growth decreased to a level of as low as 5 Gy ($P < 0.01$, *t*-test) (Figure 12). The phenomenon that x-ray irradiation-induced cytotoxicity was significantly enhanced by GSH depletion implies the involvement of ROS induction. This also confirmed the hypothesis proposed in the previous section that the lower ROS levels in Sf9 cells, either the background or induced, might somehow contribute to the higher resistance of insect cells compared to human cells.

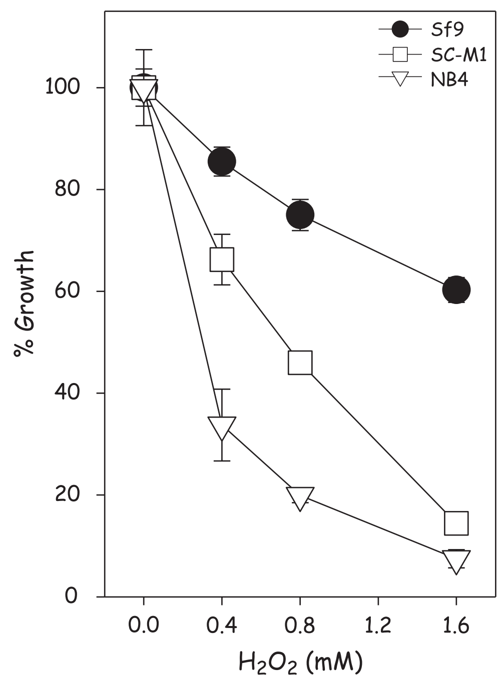


Fig. 10. Comparisons of hydrogen peroxide-induced cytotoxicity among the insect Sf9, human gastric SC-M1 and human promyelocytic NB4 cell lines.

Insect Sf9 and human SC-M1 cells are equally susceptible to N-nitroso alkylating agents

Although Sf9 cells were more resistant to ROS-generating agents including x-ray irradiation and H_2O_2, they were as susceptible as human cells to the two *N*-nitroso alkylating compounds we investigated. These included *N*-nitroso propoxur, a derivative of the insecticide, propoxur and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a well-studied human carcinogen. Both of them are known to insult human cells by forming O6-methylguanine DNA adducts (42). The dose–response curves of Sf9 and SC-M1 cells to treatment with *N*-nitroso propoxur shown in Figure 13 (left panel) indicate that

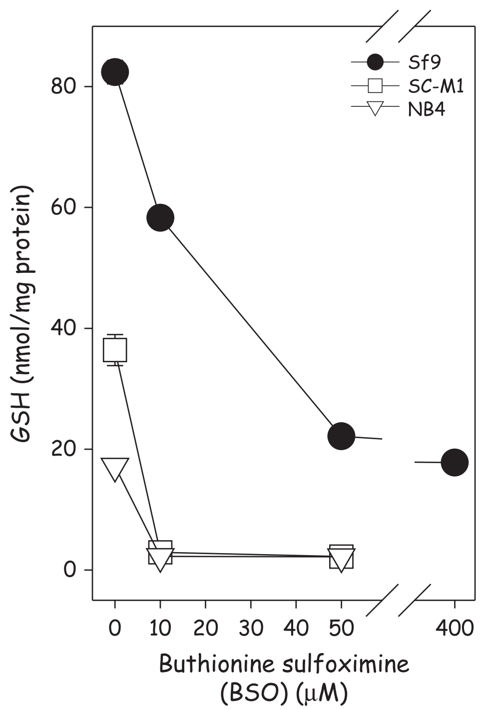


Fig. 11. Effect of BSO on intracellular GSH contents in the insect Sf9, human gastric SC-M1 and human promyelocytic NB4 cell lines.

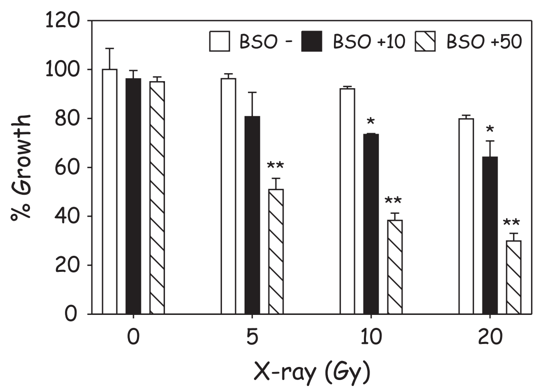


Fig. 12. Effect of BSO treatment on x-ray irradiation-induced cytotoxicity in insect Sf9 cells.

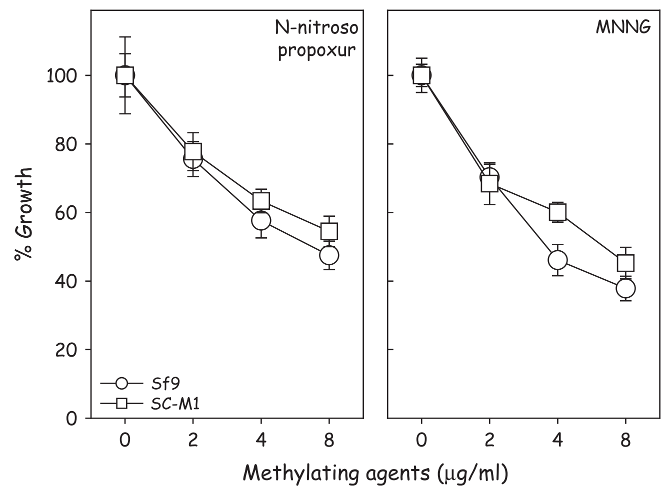


Fig. 13. Cytotoxicity of methylating agents, *N*-nitroso propoxur (left panel) and MNNG (right panel), to insect Sf9 and human gastric SC-M1 cells.

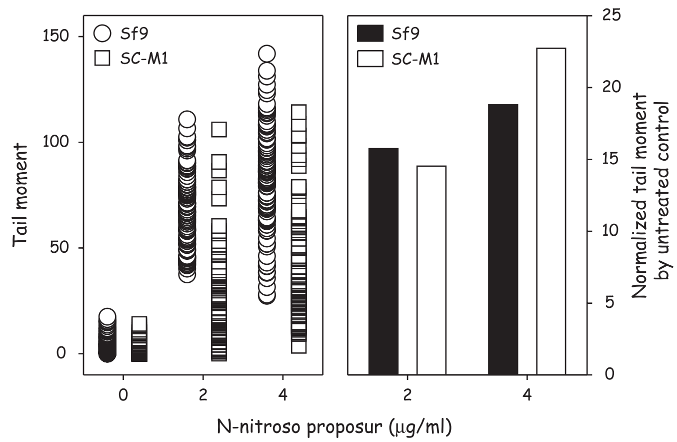


Fig. 14. Comparisons of DNA damage induced by *N*-nitroso propoxur between insect Sf9 and human gastric SC-M1 cells.

Sf9 cells were not more resistant than SC-M1 cells as they were to x-ray irradiation and H_2O_2. This was also true for treatment with MNNG (Figure 13, right panel). The results of DNA damage induced by *N*-nitroso propoxur in Sf9 and SC-M1 cells, as shown by the comet assay, are pertinent to those of growth inhibition (Figure 14, left panel). In treatments with *N*-nitroso propoxur, an increment in the median tail moment induced in Sf9 and SC-M1 cells did not significantly differ, at 15.7- versus 14.5-fold for treatment with 2 μg/ml and 18.8- versus 22.7-fold with 4 μg/ml, respectively, (Figure 14, right panel). Insect Sf9 and human SC-M1 cells had similar susceptibilities to the cytotoxicity and degree of DNA damage induced by *N*-nitroso alkylating agents.

Insect Sf9 cells are deficient in several DNA repair enzymes

Although Sf9 and SC-M1 cells were similar in *N*-nitroso propoxur- and MNNG-induced growth inhibition and DNA damage, they greatly differed in the expression of enzymes required to repair DNA damage induced by these two *N*-nitroso alkylating agents (Figure 15). *N*-nitroso propoxur and MNNG induce cytotoxicity mainly through the formation of O6-methylguanine adducts on DNA (42,43). The induction of such adducts can be prevented by a specific enzyme, MGMT, which transfers the methyl group from O6-methylguanine to a cysteine residue within the protein (43,44). The removal of O6-methylguanine adducts from DNA depends on the total number of MGMT in cells. Western analysis in our studies indicated that the MGMT protein was consistently detected in SC-M1 but not Sf9 cells. Expression of MGMT protein in insects has been reported in *Drosophila* (45) and documented as a protein with an MW of ∼19 kDa, which is close to the marker antigen we used in this study. However, we were unable to detect the expression of MGMT in Sf9 cells. The lack of MGMT probably leads the O6-methylguanine adduct in Sf9 cells to pair by mistake with thymine instead of cytosine during DNA replication. This incorrect pairing is recognized by the mismatch repair enzyme, MutSα, a heterodimer composed of MSH2 and MSH6 (44). In our results, expressions of MSH2 and MSH6 enzymes were observed in SC-M1 cells but were absent from Sf9 cells. There was a clear band of protein shown

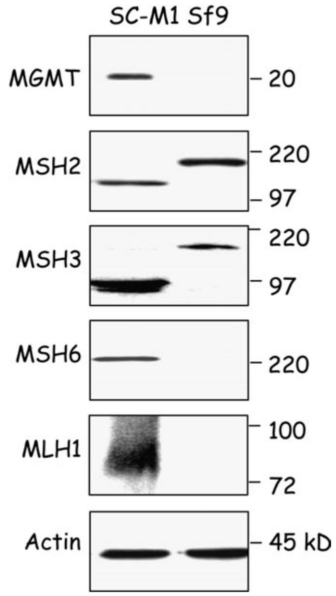


Fig. 15. Comparisons of major enzymes involved in the repair of DNA damage induced by the formation of O6-methylguanine adducts in insect Sf9 and human gastric SC-M1 cells.

in Sf9 cells at the position approximately twice the molecular weight (MW) of that of the MSH2 (102 kDa) antibody used in the Western analysis. However, that protein band did not seem to be a dimer of MSH2 since the SDS–PAGE protocol in our experiment did not allow the dimer to remain intact on the electrophoresis gel. A phenomenon similar to that of the expression of MSH2 also was found in the Western analysis of MSH3. The dimer of MSH2/MSH3, which recognizes MutSβ, was a less abundant factor in the miss-matched repair (MMR) system and has not been implicated in DNA damage signalling (46). MutL is another essential MMR protein mediating between MutS and MutH (47). MLH1, the major component protein of three distinct MutL heterodimers, which is significantly expressed in SC-M1 cells, was not detected in Sf9 either. Overall, DNA repair enzymes that are involved in removing O6-methylguanine adducts and repairing damage thereafter were proficient in SC-M1 but deficient in SF9 cells. In fact, the deficiency in MMR family proteins is not restricted to cells isolated from the species *S. frugiperda*. The genome of *Drosophila melanogaster* contains orthologues of MSH2 and MSH6 but lacks genes to encode MSH3, MSH4 and MSH5 (48).

Discussion

Adult insects are known to be more resistant to ionizing radiation than vertebrates (8). This phenomenon was at first attributed to the low level or lack of cell division in most cells comprising adult insect species. Investigations using cell lines, however, revealed that, with about the same mitotic indices (49), cells isolated from insects are more resistant to the lethal effect of irradiation than those from mammals (11,13,50). The distinction in proliferative status did not seem to play a significant role in verifying the differential radioresistance between insects and vertebrates. Factors other than a proliferation difference have been proposed to elucidate the mechanism of the high radioresistance in insects and insect cell lines (11). Chromosome size or DNA content per chromosome, in particular, has been suggested to be important in determining insect cell radiosensitivity (11). However, the DNA content of cells isolated from the mosquito *Aedes albopictus* is reported to be comparable to that of mammalian cells (51), yet they are more resistant to mutagenic insults than mammalian cells (41,49,52). By and large, cells isolated from lepidopteran insects were found to be more radioresistant than those from other insect orders (11,16). The holokinetic nature of chromosomes in lepidopterans, which provides cells with fragmented chromosomes a better chance to survive the severe screening of the cell division process, has been proposed as one of the possible mechanisms for their higher radioresistance. This hypothesis, nevertheless, became less convincing when cells from hemipterans, which also have holokinetic chromosomes, were found to be much less radioresistant than lepidopteran cells. Results from a series of studies in the TN-368 cell line isolated from the cabbage looper, *T. ni*, led Koval and colleagues to conclude that the lower sensitivity to DNA insults and higher efficiency of DNA repair are major factors determining the higher radioresistance in insect than mammalian cells (13,15,50,53,54). Development of insect cell culture practices provided useful tools for elucidating the radioresistance mechanism. In spite of this, research in this field was not as active as had previously been expected. In this report, another lepidopteran cell line, Sf9, isolated from the fall armyworm, *S. frugiperda*, was used. Compared with TN-368, investigations on the radioresistance of Sf9 cells are rare (16). Our results found that Sf9 cells also possess very much higher resistance to x-ray irradiation than several human cell lines.

The dose-response curve of cell lines to x-ray irradiation showed that >90% of Sf9 cells survived treatments that killed >50% of human cells. It seems that up to the dose limit that kills most human cells, an increase in irradiation dose is unable to overcome the radioresistance of Sf9 cells. This phenomenon is comparable to the concept of oncogenic resistance proposed by Blagosklonny (55). The development of oncogenic resistance is due to alterations in regulation of apoptosis so that the downstream pathways of apoptosis are blocked. Our results from the DNA ladder assays and annexin V staining technique, indeed, showed that apoptosis induced by x-ray irradiation occurred in human cells but not in Sf9 cells. The phenomenon is analogous to oncogenic resistance observed in Sf9 cells and confirms the suggestion that radioresistance in insect cells is an intrinsic nature of the insect (54).

Parallel to the phenomenon observed in TN-368 cells as described previously, our results indicated that x-ray irradiation induced much less DNA damage in Sf9 than in human cells. Growth inhibition and apoptosis induction were significantly correlated with the degree of DNA damage among treated cells. The comet assay allowed us to estimate the efficiency of DNA repair through the scoring of the DNA tail moment which disappeared during the post-irradiation period; it revealed that Sf9 cells decreased their DNA damage at a rate much faster than human cells. In addition, repair of DNA damage did not seem to be homogenously implemented for every x-ray-irradiated human cell. After treatment removal, a certain portion of damaged human cells retained their tail moments at a level significantly higher than others, indicating the lack of comprehensive repair of their damaged DNA. The percentage of human cells with damaged DNA left un-repaired increased as the post-incubation period was extended. This unique phenomenon was not observed in Sf9 cells and also does not seem to have been described previously by other authors.

Whether this is the portion of cells that are subsequently destined to undergo apoptosis awaits further investigation.

It is well established that irradiation treatment generates intracellular ROS, which in turn act as common mediators for apoptosis (56). However, radioresistance in insect cells has never been correlated with the induction of apoptosis and/or ROS in previous reports. Our investigation revealed that Sf9 cells had lower background intracellular ROS levels. Although intrinsic factors such as the uptake, ester cleavage or efflux of DCFH-DA dye might vary among cells of different origin, thereby hinders the unbiased measurement of background ROS, the ROS induced by treatments with x-ray irradiation were also lower in Sf9 cells than in the other human cell lines studied. The significance of the low intracellular ROS to the radioresistance of insect cells was corroborated by the experiment with H_2O_2, which is a typical ROS-generating agent. Our investigation showed that Sf9 cells were more resistant to and induced less intracellular ROS after treatment with hydrogen peroxide than did the human cell lines. The relationship between ROS and insect radioresistance became even clearer when the intracellular GSH level was investigated. GSH is known to be a powerful antioxidant which reduces intracellular ROS and is frequently associated with a decrease in ROS-induced cytotoxicity (40). In Sf9 cells, the background GSH level was more than twice as high as in the human cells. Deprivation of GSH by pre-treatment with the GSH inhibitor, BSO, significantly decreased the radioresistance of Sf9 cells. Moreover, the concentration of BSO needed to significantly decrease the GSH level of Sf9 cells was $>$40 times higher than that in the human cells. All those results obviously indicate that the higher level of background antioxidants and lower level induced by ROS play significant roles in determining the higher radioresistance of insect cells.

In addition to x-ray irradiation, insect cells were found to be more resistant than human cells to other mutagens, including UV (53), bleomycin (49), streptonigrin (52) and possibly many others as well (41). Our investigations on the cytotoxicity of *N*-nitroso alkylating agents to insect and human cells, however, showed a different picture. The *N*-nitroso alkylating agents used in this report were found to be equally toxic to Sf9 cells and to the SC-M1 human gastric cell line. The degree of DNA damage induced by these 2 *N*-nitroso alkylating agents, which are known to form O6-methylthioguanine adducts on DNA, was similar between Sf9 and SC-M1 cells. Despite the similarities in cytotoxicity and DNA damage induced in these two cell lines, SC-M1 cells were proficient while Sf9 cells were deficient in enzymes involved in the repair of the specific DNA damages induced by those two compounds. A similar phenomenon was described in human cells with less or no MGMT activity, a deficiency in MMR enzymes, such as MutSα or MutSβ, which on the contrary, enables them to be more resistant to treatment with *N*-nitroso alkylating agents than those efficient in MMR enzymes. This is because MMR enzymes, which originally play a role in detecting mismatched DNA pairing due to O6-methylthioguanine adducts, repair DNA damage by first inducing DNA strand breaks, which sometimes lead cells to apoptosis. A lack of MMR enzymes in MGMT-deficient cells allows *N*-nitroso alkylating agent-insulted cells to escape the cytotoxicity caused by DNA strand breaks and proceed to develop a resistance to the chemicals. Whether this can explain the similar resistance in Sf9 and human cells despite their significant difference in repair enzymes awaits further investigations.

In previous reports and this communication, insect cells shown to be more resistant than human cells were mostly treated with agents capable of inducing intracellular ROS, including x-ray irradiation, H_2O_2, BSO (in this report), γ-ray irradiation (16), UV (53), bleomycin (49) and streptonigrin (52). When being treated with MNNG and *N*-nitroso propoxur, which do not induce ROS, no significant difference in resistance was found between those two cells. The similarity in resistance to *N*-nitroso alkylating agents between Sf9 and SC-M1 cells seems to highlight the decisive role of ROS in determining the higher resistance of Sf9 cells.

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